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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Martin G. Sirois, et al.
Serial No.:	09/945,131
Filed:	August 31, 2001
For:	LOCALIZED OLIGONUCLEOTIDES THERAPY FOR PREVENTING RESTENOSIS
Group Art Unit:	1635
Examiner:	Gibbs, Terra G.

DECLARATION UNDER 37 CFR 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450
U.S.A.

Dear Sir:

1. I, Dr. Martin G. Sirois, am one of the named inventors in the above-identified patent application.

2. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

3. A brief explanation of stenosis and restenosis is thought to be in order for a better understanding of the remainder of the present Declaration.

Stenosis/restenosis

4. Stenosis is a narrowing of the orifice of the vessel that occurs generally over a long period of time (can be 50 years) in human. It is generally treated by performing an angioplasty (dilatation of the blood vessel with a catheter being inserted into the vessel lumen). Restenosis is a new narrowing of the vessel orifice which can be induced as a result of the lesion caused by angioplasty

5. Precise events that conduct to the re-narrowing of the vessel were studied in animal models. Following angioplasties, the damage to endothelial cells and medial smooth muscle cells exposes the subendothelial connective tissue to blood-borne elements leading to a coordinated sequence of cellular events including: platelet adhesion/activation and aggregation, leukocyte and monocyte adhesion and transmigration, and release of mitogenic and chemotactic growth factors. These growth factors elicit a cascade of intracellular signal pathways, and overexpression of genes critical to the proliferation and migration of medial smooth muscle cells and result in intimal hyperplasia. The intimal hyperplasia process following an angioplasty can be divided in a number of waves. The first wave (0-3 days) is governed by proliferation of medial smooth muscle cells (SMC). Although a number of mitogens including the acidic and basic fibroblast growth factors (aFGF, bFGF), insulin-like growth factors (IGFs), interleukin-1, thrombin and angiotensin II stimulate SMC proliferation *in vitro* it appears that bFGF released from dying medial SMC is the predominant mitogenic factor for the first wave. It was shown that treatment with anti-bFGF antibodies can reduce medial SMC proliferation by more than 80%, but does not prevent intimal

thickening. The second wave (3-14 days) consists of the migration of medial SMC across the internal elastic lamina to the intima. If the medial SMC proliferation (first wave) is not a prerequisite for the induction of intimal thickening, it appears that the migration of medial SMC is absolutely required for the development of intimal hyperplasia and the predominant mediator is platelet-derived growth factor-BB (PDGF-BB). When rats are treated with polyclonal antibodies to all forms of PDGF, the intimal hyperplasia mediated by balloon catheter injury is inhibited, even though the first wave of proliferation is not prevented. Experiments in the rat carotid injury model showed that a local bolus endovascular delivery of AS-PDGFR-β blocked the migration of vascular smooth muscle cells, and intimal hyperplasia by 60% but also doubled the reendothelialisation process as compared to control animals that received a carotid vascular injury without AS-PDGFR-β treatment.

Prior art

6. Antisenses against *c-myb*, *c-myc*, *PCNA*, *cdc2/cdk2* have been shown to produce a significant reduction in vascular smooth muscle cells (SMC) proliferation and intimal tickening.

7. Furthermore, these genes are all implicated in the cell proliferation cycle. As such, they induce proliferation of all cells including endothelial cells. Hence, blocking expression of these genes therefore would prevent proliferation of every cell including **endothelial cells**. Antisenses against these genes therefore would not favor reendothelialisation but rather prevent it.

8. Application '845 simply suggests using antisense against PDGFR-β to inhibit smooth muscle cell growth or recruitment of white cells and

resultant fibrosis (see page 18, lines 18-126; page 19, lines 19-21; and page 20, lines 22 to 28 of '845). No experimental data are provided showing the production of these antisenses.

9. The Applicants are the first to report an inhibition of the expression of PDGFR-β by an antisense oligonucleotide.

10. At the time of filing of the present application, namely on 31 August 2001, it was not known (other than by the Applicant's publication less then one year prior to 31 August 2001) that PDGFR-β played a role in endothelial cell growth, proliferation or function.

11. Prior to making antisenses against PDGFR-β and testing them in a rat vascular model as reported in the present application and observing its effect on endothelial cells, it could not be expected that these antisenses could improve reendothelialization and vascular function. These effects had not been observed previously and were surprisingly shown to assist in the inhibition of restenosis.

Additional results in a porcine model

12. Once antisenses were produced and shown to improve reendothelialization and vascular function, a person of ordinary skill in the art could through routine methods identify efficient antisenses in other species and reasonably expect that these antisenses could effectively work in other species. This affirmation is supported by the following paragraphs.

13. PDGF receptors and ligands were known to be present in normal arteries of rat, pig, baboon and human, in human atherosclerotic plaques and in injured rat and human arteries.

14. At the time of filing of the present application, the nucleotide sequences of human, rat, and mouse PDGFR-β were known. Hence, the mouse PDGFR-β was published on GenBank™ as early as March 30, 1995 under accession number NM_008809; and that for human PDGFR-β was published as early as November 17, 2000 under accession number XN_003790.

15. There is significant homology between the pig, rodents (mice and rats) and human PDGFR-β nucleotides sequences. A comparison of the PDGFR-β nucleotides sequences of the mammalian species that are most distant phylogenetically, namely mouse and human. The homology is of 71% between human and mouse for the full cDNA while it is of 83% for the coding portion of the DNA starting at the initiation codon up until nucleotide 3718 inclusively of the human sequence. Note that although the alignment of rat/human was not provided, its homology is at least 95% identical to that of mouse The homology between the fragment of the sequenced pig cDNA and the corresponding human sequence is of 100%. The alignment for the PDGFR-β gene human, pig (partial sequence) and mouse is attached.

16. It was reasonable to predict that if antisenses could successfully be used in rat to inhibit neointimal hyperplasia, it could also be successfully used for this purpose in other species including pig and humans.

17. Partial pig PDGFR-β cDNA was identified and cloned through routine methods as follows:

18. Based on the previously reported sequences of human and mouse PDGFR-β cDNA (Genbank access codes: Human sequence:

XM003790; Mouse sequence: NM008809), two oligonucleotide primers covering the ATG initiation codon, 5'-GCC CAC ACC AGA AGC CAT CAG CAG-3' in the 5' non-coding region (-34 to -11) and 5'-AAC CCG AGC AGG TCA GAA CGA AGG-3' in the 3' coding region (+189 to +165) (Genosys Inc.) were synthesized. Total RNA was isolated from cultured porcine aortic smooth muscle cells (PASMC; passage 4 to 8) using TRIZOL™ reagent (Gibco Inc.) by following manufacturer's instructions. cDNA was prepared by RT-PCR using 5ug of total RNA as a template for random hexamer-primed first strand cDNA synthesis and MoMuLV™ reverse transcriptase (NEB™ Biolab). Polymerase chain reaction (PCR) was performed using TAQ DNA polymerase (Promega™) with the above-mentioned primers for 30 cycles (denatured at 94°C for 1 min; annealed at 60°C for 30 sec; extended at 72°C for 2 min). After electrophoresis, the RT-PCR yielded a 204 bp product which was treated with the Klenow fragment of DNA polymerase I and T4 polynucleotide kinase, and ligated in pBluescript™ II SK (Stratagene) at the SMA I site. This construct was cloned in competent *Escherichia coli* Dh 10b cells, and were grown on plastic plates containing YT media, X-gal (0.8 mg/plate), IPTG (0.8 mg/ml) and ampicillin (0.2 mg/plate). Ampicillin-resistant clones were isolated, grown in 2 ml of YT media and Minipreps™ were performed as described previously (Maniatis). Sequence analyses of isolated DNA from positive clones was carried out on both alkaline-denatured template strands using Sequanase™ 2.0 (U.S. Biochemical Corp.). These manipulations were performed in duplicata.

19. It is routine in the art to identify antisense oligonucleotides for genes for which the sequences are known using computer modelizations.

20. When fed a gene sequence, these softwares are able in about 1 hour, to provide a list of the oligonucleotides that are the most likely to be efficient antisenses against that gene by determining for instance the mRNA regions that most likely are accessible, which antisense sequences are less likely to form hairpin structures, etc. The antisense oligonucleotides so provided may then be tested for activity *in vitro* and the best candidates are selected for *in vivo* tests.

21. *In vivo* tests were thus performed on 28 pigs with the best oligonucleotide candidates and were completed over about 12 months.

22. It is to be noted that although the pig model used does not perfectly reproduce restenosis occurring in human[1], it is a very good model to predict the physiological events that will occur in human as a response to the administration of a restenosis inhibitor. Indeed, pigs and humans are very similar in a number respects which are all relevant for restenosis. For instance, atherosclerosis develops similarly in pigs and humans; the quantity and quality of lipids produced are similar in pigs and human; in both species, there is a transient (of about 45 days) surexpression of PDGFR-β receptor following vascular aggression Finally, pigs are one of the only animal models (with primates) that can be used to perform a coronary vascular lesion, thereby closely mimicking angioplasty performed in patients suffering from stenosis.

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[1] In our animal models, the blood vessel used to test the candidate anti-restenosis compound, is a healthy vessel that is injured through the insertion of a catheter for instance to reproduce damages caused by an angioplasty The consequence of the injury on the animal's blood vessel may therefore not technically be called "restenosis" because no stenosis existed prior to the induced injury. The consequence of the injury is therefore called intimal hyperplasia but it is very similar in fact to what occurs in restenosis (SMC proliferation and migration etc..).

23. Treatment with antisense oligomers targeting pig PDGFR-β mRNA reduced the percentage of stenosis (neointima formation) in function of the internal elastic laminae (IEL) fracture (%) as compared to the saline groups. In the saline-treated group, we observed a slope of: y = 1,06x + 6.09, whereas a treatment with the AS1-PDGFR-β (y = 0,52x + 5,54) reduced the degree of the slope by 51% (P = 0,004; compared to SALINE). In addition, we observed that such treatment also favored the reendothelialisation process in injured coronary arteries. See Table 1 and Figures 1-4 attached.

24. These observations are in agreement with our previous data in which we observed in a rat carotid angioplasty model, that a single bolus endovascular delivery of AS1-PDGFR-β at the injury site reduced the stenotic process and favored the reendothelialisation process. Although the models of vascular injury and the arteries treated are different in the rats and in the pigs, it illustrates nevertheless positive similar effects regarding the potential of targeting PDGFR-β protein expression to prevent stenosis and to favor the vascular healing process.

Respectfully submitted,

March 04/2004
Date

Martin G. Sirois